Supporting Information

1. Supporting Methods

1.1. Cell Lines

SKBR3 (ATCC HTB-22), MCF7 (ATCC HTB-30) and MDA-MB-231 (ATCC HTB-26) human breast carcinoma cells were obtained from American Type Culture Collection (ATCC). SKBR3 and MCF7 are epithelial cells well known to be EMT-inducible following treatment with HRG.[1-3] MDA-MB-231 cells are mesenchymal-like cells used as a positive control.[4] Cells were maintained at 37 °C in a humidified environment with 5% CO_2 in complete media which is McCoy's 5A media (Life Technologies) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Mediatech) for SKBR3, Eagle's Minimum Essential Media (EMEM) (Life Technologies) supplemented with 10% (v/v) FBS (Mediatech), 2 mM glutamine (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich) and 1 x MEM non-essential amino acids (Sigma-Aldrich) for MCF7 and MEM alpha (Life Technologies) supplemented with 10% (v/v) FBS, characterized (Mediatech), 2 mM L-glutamine (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 1 x MEM non-essential amino acids (Sigma-Aldrich) and 1ug mL^{-1} insulin for MDA-MB-231. All experiments were performed in serum-free media to eliminate activation of the pathway by other signal transducers that may be present in serum.[5]

1.2. Reporter gene assays

Luciferase reporter gene constructs containing wild-type E-cadherin promoter sequences were a gift from E. Fearon.[6] The E-cadherin promoter region containing 3 Eboxes (from 2108 to +125 of the endogenous E-cadherin gene) were cloned into pGL2-Basic upstream of firefly Luciferase (Ecad-luc). Ebox elements in the E-cadherin promoter in Ecad-luc were mutated from 5'-CAGGTG-3' to 5'-AAGGTA-3' in EcadMut-luc. To examine the repression of the E-cadherin reporter gene construct by endogenous and EMT-induced Snail, 1 x 10^5 cells were plated in each well of a 24-well plate and allowed to adhere overnight. The media was replaced with serum-free media, and 200 ng of the E-cadherin construct (Ecad-luc or EcadMut-luc), 50 ng of the Renilla luciferase construct as a control and 40 nM of the experimental compound were transfected per well. The experimental derivatives included the Co(III) Schiff base complex (Co(III)-sb), the Ebox double-stranded oligonucleotide (ds Ebox), the Co(III)-DNA conjugate with 2-base pair substitution in the Ebox region (Co(III)-EboxMut) and the Co(III)-DNA conjugate targeted to Snail family TFs (Co(III)-Ebox). Cell lines were transfected using Turbofect Transfection Reagent (Thermo Scientific) according to the manufacturer's protocol. In each experiment the total DNA transfected in each well was equalized by the addition of noncoding DNA as pCS2+ empty vector to samples that do not contain experimental compound. Following transfection, the SKBR3 or MCF7 cells were treated with 20 ng mL^{-1} of HRG for 24 or 48 h, then cell extracts were prepared using Passive lysis buffer (Promega).

Firefly luciferase and Renilla luciferase activity were determined using the Dual-Luciferase Reporter assay kit (Promega) on a GloMax 96 Microplate Luminometer (Promega). The results were normalized by dividing firefly luciferase activity by Renilla luciferase activity and reported as fold inductions of the vehicle control. These values were averaged over three replicates and reported with the standard error. Statistical analysis was performed on the means using the Student's t-test where *$P<0.05$ and **$P<0.005$.

1.3. Western Blot Analysis

SKBR3 or MCF7 cells were plated at a density of 2.5 x 10^5 cells in each well of a 6-well plate and allowed to adhere overnight. The media was replaced with serum-free media, and 40 nM of the experimental compound [Co(III)-sb, ds Ebox, Co(III)-EboxMut and Co(III)-Ebox] were transfected per well using Turbofect Transfection Reagent (Thermo Scientific) according to the manufacturer's protocol. Following transfection, the cells were treated with 20 ng mL^{-1} HRG for 24 or 48 h, and cell extracts were prepared using a cell lysis buffer (50 mM Tris-HCl (pH=7.4), 1% (v/v) NP40, 0.25% (w/v) sodium deoxychlorate, 100 mM NaCl, 1 mM EGTA and 1 mM NaF). Samples were denatured in Laemmli buffer and resolved by SDS-PAGE on a 10 % (w/v) acrylamide gel. Each lane of the gel was loaded with 10 μg of total protein. After transferring to a nitrocellulose membrane (Whatman), specific proteins were detected using anti-cytokeratin-18 (Ab-2) mouse mAb (1:200) (Calbiochem), anti-Snai1 rabbit mAb (1:1000) (Cell Signaling Technologies) and anti-GAPDH rabbit mAb (1:500) (Sigma-Aldrich). ECL anti-rabbit IgG HRP-linked antibody (1:3000) (Amersham) and anti-mouse IgG (H+L) HRP conjugate (1:3000) (Promega) were used as secondary antibodies.

The immunoreactive bands were detected using a SuperSignal West Pico Chemiluminescent Substrate (Pierce). Band images were obtained by using ChemiDoc XRS+ (Bio-Rad) and band intensity analyzed by ImageJ software (NIH). Images presented are a representative replicate of triplicate samples. Band intensity values were normalized to the GAPDH signal in each lane. The percentage of protein expressed is the normalized intensity of each treatment divided by the normalized intensity of the untreated lane. These values were averaged over three replicates and reported with the standard error. Statistical analysis was performed on the means usihe Student's t-test where *P<0.05 and **P<0.005.

1.4. Immunofluorescence

SKBR3 or MCF7 cells were plated at a density of 5 x 10^5 cells in each well of a 6-well plate onto glass coverslips and allowed to adhere overnight. 40 nM of Co(III)-Ebox was transfected per well using Turbofect Transfection Reagent (Thermo Scientific) according to the manufacturer's protocol. Following transfection, the cells were treated with 20 ng mL^{-1} of HRG for 24 or 48 h, and fixed at 4 °C for 10 mins using 3.7% (w/v) paraformaldehyde in PBS. The cells were then blocked for 1 h in 6% (w/v) BSA with 0.25% (v/v) TritonX-100 in PBS. The coverslips were washed in PBS (3 x 10 mins) and incubated with anti-fibronectin mouse mAb (1:1000) (Thermo Scientific) in 3% (w/v) BSA with 0.25% (v/v) TritonX-100 in PBS for 1 h. The coverslips were washed in PBS (3 x 10 mins) and incubated with Cy5-conjugated anti-mouse secondary antibody (1:500) in 3% (w/v) BSA with 0.25% (v/v) TritonX-100 in PBS for 1 h. The coverslips were washed in PBS (3 x 10 mins) and mounted onto a glass microscopy slide using ProLong Gold antifade agent with DAPI (Invitrogen) and allowed to cure overnight.

Cells were imaged on a DeltaVision Deconvolution Microscope (Applied Precision) equipped with a Coolpix HQ Camera (Nikon) using a PlanApo N *60x/1.45 Oil* objective. The images were deconvoluted and analyzed using the DeltaVisionSoftWoRx™ software. At least 3 images were taken per slide and repeated on at least 3 separate occasions. Images presented are a representative replicate of triplicate samples. Relative quantification of the fluorescence intensity was carried out using ImageJ by drawing a 20 μm diameter circle over a representative portion of the image and measuring the integrated fluorescence intensity. Background fluorescence was measured in areas of the image that did not have cells and subtracted from the measured integrated fluorescence intensity.[7]

Measurements were taken from at least five different images in each treatment group. Statistical analysis was performed on the means using the Student's t-test where *P<0.05 and **P<0.005.

1.5. Zymography

SKBR3 and MCF7 cells were plated in complete media at a density of 2 x 10^5 cells in each well of a 6-well plate and allowed to adhere overnight. The media was then replaced with serum free media. 40 nM of Co(III)-Ebox was transfected per well using Turbofect Transfection Reagent (Thermo Scientific) according to the manufacturer's protocol and the cells were subsequently treated with 20 ng mL^{-1} HRG in each well. Aliquots of the new media were taken after 0, 24 and 48 h. Samples were denatured and resolved on a 1% (w/v) gelatin zymography gel cast in acrylamide. Each lane of the gel was loaded with 10 ug of total protein. The SeeBlue® Plus2 Pre-Stained Standard (Invitrogen) was used as a mass marker. The gel was placed in renaturing buffer (50 mM Tris-base, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 2.5 % (v/v) TritonX-100, pH=7.4) for 1 h at room temperature then placed in developing buffer (50 mM Tris-base, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.01% (w/v) NaN_3, pH = 7.4) at 37 °C for 12h. The gel was stained for 30 mins using a Coumassie Blue stain (0.5 g Coumassie Brilliant Blue R250 dissolved in 45 mL MeOH, 45 mL H_2O and 10 mL glacial acetic acid) then de-stained with 30% (v/v) acetic acid and 10% (v/v) ethanol in water for 1 h.

1.6. Scratch wound assay

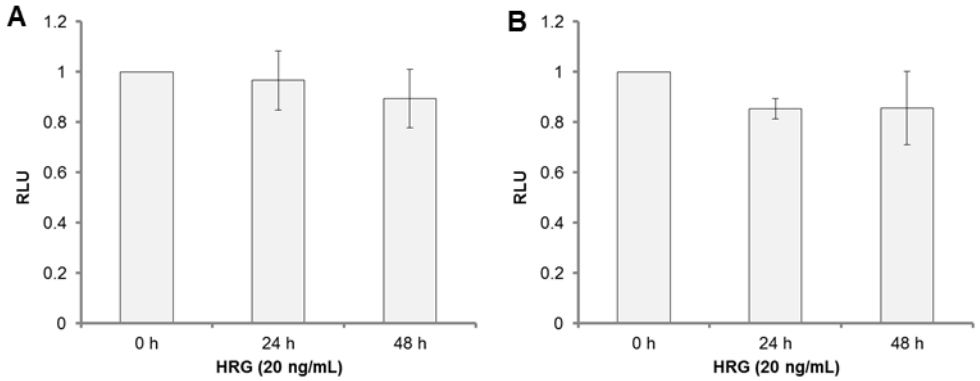
SKBR3 and MCF7 cells were plated at a density of 2 x 10^5 cells into each well of a 48-well plate and allowed to adhere overnight. The media was replaced with serum free media, serum free media containing 20 ng mL^{-1} of HRG or serum free media containing 20 ng mL^{-1} of HRG and 40 nM of Co(III)-Ebox complexed to Turbofect Transfection Reagent (Thermo Scientific). The cells were allowed to grow to confluency over 2 days, at which point a scratch wound was created in the cell monolayer using a 2 μL pipette tip. A razor was used to mark the underside of the plate perpendicular to the scratch wound as a reference point for imaging. Images of the scratch wound were obtained every 3 h for the first 9 h, then at 24 and 48 h on a Invertoskop 40C microscope (Zeiss) equipped with a ProgRes®C3 camera (Jenoptik) using a CP-ACHROMAT 5x/0.12 objective. The data was processed by measuring the width of the scratch wound at 10 different places and representing as a percentage of the width at t=0. Experiments were conducted in triplicate on at least three separate occasions. These values were averaged and reported with the standard error.

1.7. Transwell migration and invasion assays

The transwell migration and invasion assays were performed using the Corning® Transwell® 96 well permeable supports according to the manufacturer's instructions. SKBR3, MCF7 and MDA-MB-231 cells were plated in a T75 flask and allowed to grow to ~50% confluency. The media was changed to serum-free media, and the cells were allowed to grow overnight, then removed from the flask using Accutase®. The cells were counted using a Guava easyCyte® flow cytometer (Millipore) and 5 x 10^4 cells were plated in each well of the Corning® Transwell® 96 well permeable support insert in 50 μL serum-free media with or without 20 ng mL^{-1} HRG and/or 40 nM of Co(III)-Ebox complexed to Turbofect Transfection Reagent (Thermo Scientific). For invasion assays, 35 μL of 0.25 μg μL^{-1} Cultrex® Basement Membrane Extract (Trevigen) diluted 1:4 with appropriate media was plated in each well of the insert and allowed to gel at 37 °C prior to plating cells. 150 μL of complete or serum-free media was placed in each well of the receiver plate. The cells were allowed to incubate for 48 h, after which the inserts and receiver wells were gently washed with PBS. The cells on the underside

of the insert were dissociated with 2 mM Calcein-AM (AnaSpec) in Accutase®, and the fluorescence emission at 520 nm in each well was measured using a Synergy 4 microplate reader (BioTek), exciting at 485 nm. These values were averaged over three replicates and reported with the standard error. Statistical analysis was performed on the means using the Student's t-test where *P<0.05 and **P<0.005.

2. Supporting Figure



Supporting Figure 1. (A) SKBR3 and (B) MCF7 cells tranfected with EcadMut-luc did not show a decrease in E-cadherin expression in response to HRG.

3. Supporting Movies

Supporting_Movie_1.avi (separate attachment)

Supporting Movie 1. Reconstructed z-stack image of spheroid embedded in BME treated with HRG and allowed to grow over 12 days. The spheroids were fixed and stained with DAPI prior to imaging.

Supporting_Movie_2.avi (separate attachment)

Supporting Movie 2. Reconstructed z-stack image of spheroid embedded in BME treated with HRG and Co(III)-Ebox and allowed to grow over 12 days. The spheroids were fixed and stained with DAPI prior to imaging.

4. Supporting References

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